BRAUN

The Hydrolysis of Proteids

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THE HYDROLYSIS OF PROTEIDS

...BY...

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THESIS

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THIS IS TO CERTIFY THAT THE THESIS PREPARED UNDER MY SUPERVISION BY

Walter C. E. Braun (Under Dr. W. M. Dehn)

ENTITLED The Hydrolysis of Proteids.

IS APPROVED BY ME AS FULFILLING THIS PART OF THE REQUIREMENTS FOR THE DEGREE

OF Bachelor of Arts in Chemistry

H. S. Grindley

HEAD OF DEPARTMENT OF

The Hydrolysis of Proteids.

Since proteids play so important a part in the chemistry of the living being, numerous investigators have tried for many years to determine the constitution of these bodies. Proteids are very widely distributed both throughout the plant and animal kingdoms and a definite knowledge of their chemistry would help greatly to understand the metabolic changes taking place in living organisms.

The elements entering into the composition of the proteids are carbon, hydrogen, nitrogen, oxygen, and sulphur. They exist in an almost constant ratio in these bodies and they probably bear a similar structural relation to each other although it has not as yet been established what this relation is. The similarity in their chemical behavior tends to confirm the above statement.. To illustrate this structural relation between the different proteids it may be said that all protein bodies possess neither strongly acid nor strongly basic properties but form salts with both acids and bases although their affinities for either are very weak. Another characteristic of proteids is the ease with which they coagulate at elevated temperatures i.e. they change from their soluble form to an insoluble one. Acids greatly accelerate this processof coagulation. The readiness with which proteids can be salted out is another peculiarity of this class of bodies and it was formerly believed that it was a characteristic of proteids only, although it has since been shown that all substances can be more or less easily precipitated by the salting-out process.

In the water-free condition, proteids are almost white, amorphous, non-hydroscopic bodies which are insoluble in the ordinary

organic solvents such as alcohol,ether,chloroform,etc. but are more or less soluble in either water or dilute acids or alkalies.[1] They dissolve in strong mineral acids with decomposition. Some proteids crystallize in the hexagonal system (Wichmann) while others form definitely shaped globulites.

The per-cent composition of crystalline serum-albumine is according to Michel (1) as follows:

C	53.08 %
H	7.10
N	15.93
S	1.90
O	21.99

The carbon and nitrogen content in all proteids are fairly constant varying from about 52 to 55 % in the case of carbon,and from 15-19 % in the case of nitrogen.

Molecular weight determinations made by the osmotic pressure and freezing point methods give figures ranging from 5000-18,000. Of course these results mean very little,since the sources of error in their determinations are very great yet they do indicate the complexity of the proteid molecule with some degree of certainty. Because of their complexity and because of the imperfection of our methods for the quantitative estimation of the decomposition products resulting in the study of proteids,the difficulties to be overcome in an exhaustive study of these bodies are unusually great.

Several methods for the study of proteids have been followed. According to Cohnheim these are (2)

I Boiling with more or less concentrated mineral acids.

By this method are obtained, mono-amido fatty acids,diamido fatty acids etc. Most proteids yield the same decomposition products but in greatly varying quantities.

II Boiling with superheated steam at different temperatures.

III Boiling with caustic alkalies with or without increased pressure.

IV Melting with alkalies

Amido-acids as well as substances having a fecal odor were obtained. These latter compounds were found to be skatol and indol (3).

V Digestion of the proteids with ferments produced by the animal organism-trypsin.

By this method both mono-and diamido fatty acids were obtained as well as cadaverine,cystin etc.

VI Decomposition by the action of bacteria.

Putrefaction is an example of this method. Among the decomposition products obtained by this method were,skatol,indol, skatol-acetic acid,cresol,phenol,higher fatty acids,ptomaines as cadaverine and putrescine,mercaptans.

VII Methods based upon natural digestion

Here again both mono-and di amido fatty acids are formed as well as guanidine,arginine,Phenyl alanine tyrosin etc. In all of the above methods,ammonia and carbon dioxide are liberated,probably as secondary decomposition products. The reaction in the above cases progress as follows: first the proteids decompose into albuminates and albumoses etc.

and these in turn break up into the crystalline substances which have a comparatively simple molecular structure. The crystalline decomposition products that have been found in the study of proteids are the following.

1 Fatty amido acids up to caproic acid. In these the amido group holds the alpha position.

2 Amido acids of the aromatic series.

3 Diamido acids of the fatty series.

4 Fatty acids to caproic acid as well as succinic, glutaric, plenyl propionic, p oxy-phenyl alanine, skatol-acetic acid and skatol-carbonic acid are also found.

5 Some fatty acids are present whose amido derivatives are not found such as lactic, B oxy-butric acid etc.

6 Aromatic compounds, such as phenol, kresol, benzoic acid, skatol, indol and pyrrol .

7 A piperazine derivatives whose formation is probably due to a secondary reaction.

8 Simple compounds as carbon dioxide, ammonia, oxalic acid, sulphuretted hydrogen.

9 Sulphur compounds as mercaptans, ethyl sulphide, thio-lactic acid, thioglycollic acid, cystine etc.

10 Sugar like substances as Glucosamine.

11 The unknown substance tryptophane.

Some of the above compounds are doubtless due to secondary reaction such as ammonia, carbon di oxide etc. In order to determine which of the above are due to primary decomposition and which are due to secondary reactions, the

digestion of the proteids with trypsin was resorted to because this reaction can be easily regulated. The results of such studies are that the following compounds may be considered as being primary decomposition products.

I Glycocoll
II Leucine
III Asparagine
IV Glutaminic acid
V Phenyl alanine
VI Tyrosine
VII Skatol amido acetic acid
VIII Diamido acetic acid
IX Diamido caproic acid
X Histidine
XI Arginine
XII Carbohydrates as Glucosamine
XIII Cystine and other Sulphur compounds.

From the data so far given, the following conclusions have been drawn: there are contained in the protein molecule three different kinds of nuclei vis. the aliphatic, to which belong the mono and diamido fatty acids and arginine; the aromatic which manifests itself in phenyl alanine and tyrosine and finally, a heterocyclic structure from which the indol compounds are derived. The primary substance representing this last group has not as yet been isolated. It is supposed to be skatol-amido-acetic acid. From it the already isolated compounds as skatol acetic acid, skatol carbonic acid, skatol and indol could readily be obtained by regu-

lated oxidation. Furthermore some nitrogen is liberated from proteids in the form of ammonia with more or less ease, the quantities varying with the different proteids. Considering the above results we may conclude that in the protein molecule, the larger part of the carbon is united to nitrogen, a small quantity being seemingly in a less direct relation to it; that the nitrogen exists as primary, secondary, and tertiary amines and not as nitro, nitroso or azo compounds. According to Hasse and Hausmann, some of the nitrogen exists in the CO NH_2 condition which seems to be necessary to the Biuret Reaction. Since this last mentioned reaction takes place only where two CO NH_2 groups are linked to a single carbon or nitrogen atom, it may also be safe to state that such a condition exists in the protein molecule although among its decomposition products no compounds having this particular condition, have been found. Emil Fischer has recently succeeded to synthesize several compounds by the condensation of acid amides which contain this CO NH_2 condition. He named them peptides and polypeptides (19) These substances yield a good Biuret Reaction and Fischer claims to have found such compounds among the products obtained by the partial hydrolysis of egg albumin.

As shown by Löw (17) and Hausmann (18) the non-existence of aldehyde or ketone groups has been proven. In neutral solution free hydrogen and hydroxyl ions seem to exist, giving the proteids their slightly acid and basic properties. The sulphur of the protein molecule seems to exist in two different conditions some of it being split off as H_2S by means of K OH while some of it remains unchanged.

As to the absolute structure of proteids, very little can be said. Levene [20] claims proteids to be either condensation products of the various albumoses or of the simple crystalline substances.

The object of this paper is to consider the decomposition products of the proteids obtained by hydrolizing with concentrated hydrochloric acid. In 1820, Braconnot hydrolized gelatine with sulphuric acid and discovered glycocoll . Later in 1872 Hlasiwetz and Habermann hydrolized albumine with concentrated HCl and in 1879 Horbaczewski and especially R.Cohn in 1896 determined the quantitative relations of the decomposition products obtained from Caseine. Finally Emil Fischer took up the work and hydrolized Gelatine,[4] silk glue[5] and Caseine [6] and incidentally worked out a better quantitative method for the estimation of the decomposition products i.e. by the conversion of the amido acids into their Ethyl Esters which have definite boiling points under diminished pressure [6], [7]

The final products of decomposition as obtained by the hydrolysis of proteids by means of concentrated HCl are the following;

I Glycocoll- Amido acetic acid. Found in Gelatine in large quantities and in true proteids in smaller quantities.

II Leucine- Amido caproic acid- This compound is found in all proteids in large quantities. According to Cohn 40-50% of all crystalline compounds obtained, is leucine.

III Asparaginic acid- Amido Succinic acid.
This acid was first found in plant proteids but later in animal proteids as well.

IV Glutaminic Acid- Alpha amido glutaric acid. According to Cohn, 30 % of the crystalline decomposition products of casein is glutaminic acid.

V Di amido acetic acid was found by Drechsel in only small quantities in the products obtained from casein.

VI Lysine- Di amido caproic acid-
This substance was discovered by Drechsel and found repeatedly by Schultze, Hedin and Kossel. According to Erlinger it is supposed to be the mother substance of cadaverine (penta-methylene diamine). Best authorities claim that it is always associated with arginine though only in small quantities.

VII Arginine Guanidine-amido valeric acid.
This compound was first discovered by E.Schultze and E.Steiger [8] in the extract from lupine sprouts. Later S.G.Hedin [9] prepared arginine from lysatinine, showing that this substance, discovered by Drechsel was not a single compound but a mixture of arginine and lysine. The structure of arginine was solved by Schultze [10] resulting in its synthesis [11]. Its structure is represented by the following formula:

$$NH = \overset{NH_2}{\overset{|}{C}} - NH - CH_2 - CH_2 \quad CH_2 - \overset{NH_2}{\overset{|}{CH}} - COOH$$

Arginine is considered to be the mother substance of ornithine and tetra methylene di amine as well as of urea. The greater part of the urea found in human urine is supposed to be due to the decomposition of arginine. E.Schultze [12] found that arginine, when treated with barium hydrate, decomposed as follows:

$$C_6H_{14}N_4O_2 + H_2O = C\begin{matrix} NH_2 \\ =O \\ NH_2 \end{matrix} + C_5H_{12}N_2O_2$$

urea ornithine

VIII <u>Histidine</u> (unknown constitution) was discovered by Kossel [13] Lysine, arginine and histidine are called "hexon bases" by Kossel [14] and are supposed to play a special part in the protein molecule.

IX <u>Phenyl alanine</u>- Phenyl amido propionic acid. This substance is evidently a constant product in the decomposition of proteids having been found separately by Schultze and by Pick in vegetable proteids.

X <u>Tyrosine</u>- Para-oxy phenyl propionic acid. Tyrosine was found in casein (4.5%) as well as in other proteids although in smaller quantities. Gelatine does not yield this compound on hydrolysis.

XI <u>A Derivative of Piperazine</u> - Di butyl dietlyene diamine.

XII <u>Ammonia</u> is formed in the hydrolysis of all proteids though in greatly varying quantities. 13.37% of the total nitrogen contained in casein is changed to ammonia[15] while serum-albumine only yields 0.34% of its nitrogen

towards the formation of ammonia.

XIII Oxalic acid was found in very minute quantities according to Cohn.

XIV Acetone was qualitatively identified by Cohn though no quantitative determinations were attempted.

XV Carbon dioxide has repeatedly been found.

XVI Sulphuretted Hydrogen was detected by Hlasiwetz, Habermann and Cohn.

XVII Thiolactic acid was found among the decomposition products of Keratine by Baumann and Sutter (16).

XVIII Ethyl Sulphide was found by Drechsel and considered by him to be a constant decomposition product of proteids.

XIX Cystine has been found among the products yielded by horny substances (Keratine) but has not as yet been found in connection with true proteids.

XX Glucosamine has been found in the so-called Glyco-proteids but probably exist also in ordinary proteids.

XXI Melanoidic acid is considered to be an animal dye by Schmideberg. It is precipitated on hydrolizing proteids with strong acids as a black brown flocculent mass. Nothing very definite is known about it.

EXPERIMENTAL PART.

The experimental part of this paper is intended to show the relation of the decomposition products of the insoluble proteids of beef (mostly myosine) to the products obtained by the hydrolysis of other proteids. The method followed was essentially that of

Emil Fischer (4). In order to become familiar with this method, the experiment of Fischer's on the hydrolysis of gelatine was repeated.

Hydrolysis of Gelatine.

One Kilogram of pure gelatine was dissolved in 3 liters of cold, concentrated HCl-sp.gr. 1.2-by frequent shaking. In about an hour complete solution resulted. The solution was boiled under a reflux condenser for six hours when the light brown color of the original solution was observed to have turned a deep brown. The hydrolysis mixture was evaporated under diminished pressure to a thick syrupy consistency, dissolved in three liters of absolute alcohol and the solution thus formed was saturated with dry hydrochloric acid gas. To insure perfect esterification, the solution was heated on a water bath for one hour. It was then placed in a refrigerator for about 48 hours when a crystalline mass of glycocoll hydrochloride was found to have separated out. This was filtered off, the mother liquor was again evaporated to a thick consistency and treated with alcohol and HCl gas as before. On cooling another quantity of glycocoll was precipitated. To insure complete removal of the glycocoll, the entire process was repeated a third time. A rather small yield of crystalls was the result. The crude crystalline mass was dried and weighed and the corresponding amount of glycocoll calculated.

317 gms.= am't hydrochloride obtained.

214 gms.= corresponding am't of glycocoll.

Fischer evaporated the mother liquor under reduced pressure to a syrupy consistency and diluted it with an equal volume of water. While cooling, a strong solution of NaOH was added to neutralize the free hydrochloric acid. When this was effected, a large excess of K_2CO_3 was added to salt out the esters, which were then separated from the thick mixture by extraction with ether. This process was repeated several times. The ether extract was distilled under reduced pressure (about 8 m m) and the fractions thus obtained were saponified when the acids of the esters held in solution crystallized out.

On account of lack of time the above work was omitted in the study of this method, in order to give the subsequent work on the beef proteids more attention.

Fischer obtained the following results:

Fraction I 40-55° wt. 44gm. Alanine

Fraction II 55-80 wt. 104 gm.	(Pyrrolidine carbonic (acid, Leucine and other (lower amido acids.
Fraction III 80-100° wt. 36.5	(Pyrrolidine carbonic ((acid and Leucine.
Fraction IV 100-130° 28.5 gm.	(Aspartic acid and ((undetermined substances
Fraction V 130-160° wt. 20 gms.	(Phenyl alanine ((Aspartic acid and ((Glutamic acid.

A dark brown residue weighing 125 gms and solidifying on cooling, remained.

Calculated to per cents, Fischer gave the following results.

Glycocoll	16.5%
Alanine	.8%
Pyrrollidine Carbonic Acid	5.2%
Leucine	2.1%
Aspartic acid	.56%
Glutaminic acid	.88%
Phenyl Alanine	.40%

All of the above results with the exception of glycocoll are undoubtedly too low.

ISOLATION OF BEEF PROTEIDS.

In obtaining the insoluble proteids from beef in a pure enough form for work, the following method was followed.

12 lbs. of lean beef round was freed from all visible fat and gristle and was then ground by passing through a sausage mill twice Of the ground meat, 8.5 lbs. were extracted with water to remove the soluble proteids and flesh bases as well as some of the mineral matter. This was effected by dividing the entire mass of meat into three equal parts, placing these in 12 in. evaporating dishes and pouring about 1.5 l. of nitrogen free water upon them. The meat and water was well worked until a homogeneous mixture was obtained. This was allowed to stand for some time and was then poured upon a previously boiled filter cloth spread upon a filter rack where it was allowed to drain for several minutes. Then the filter-cloth and residue was removed from the rack and wrung as dry as possible. After this same extraction process was repeated, the filtrate from the first lot was used to extract the second and

third lots. This was repeated until eight complete extractions had been made and the filtrate after passing through all three lots was entirely colorless. The amount of water used was 26 liters. In order to remove the water from the residue, it was treated with four liters of 94% alcohol. The residue was carefully broken up to insure contact with the alcohol. After standing over night, the alcohol was removed by filtration and wringing as in the case of the water extraction. In this process some fat was also removed. To remove the remaining fat, the residue after the alcohol extraction was placed into 4 liter flasks and moistened with dry ether. Enough ether was used to leave about 1/8in. layer above the residue. After frequent shaking and after standing over night the etheral solution of fat was removed as in the case of the alcohol and the residue once more treated with ether. On removal of this second extract, the residue was almost dry and practically colorless. It was dried in the air and a sample taken for analysis. The following results were obtained.

Protein (N x 6.25)	=	94.00%
Fats	=	.35%
Ash	=	.40%
Water	=	6.45%

The entire residue weighed 1176 gms.

Corresponding amount of Proteid 1105 gms.

Hydrolysis.

The protein residue obtained as described above was divided into two equal portions and placed in two 4 liter flasks. Then 1.5-l. of HCl (sp.gr. 1.2) was added to each lot while shaking.

A gelatinous mass resulted which was well stirred with a heavy glass rod to insure contact of acid with the proteids. The mixture was shaken frequently and allowed to stand over night. In about 24 hours complete solution resulted, which was boiled for six hours under reflux as in the case of gelatine. The original brown black color of the cold solution turned to a beautiful purple and finally to a dark brown. After hydrolysis a flocculent black substance was distributed throughout the hydrolysis mixture. This was removed by filtration when it formed a black gummy mass. The remaining mother liquor had an odor resembling that of wet matches which was probably due to the presence of sulphur compounds.

The Black Residue.

The black residue obtained from the hydrolysis mixture contained carbon, hydrogen, nitrogen and sulphur and corresponds to what Schmideberg called Melanoidic acid. The amount of this substance obtained was 27 grms. Since this residue contained compounds soluble in alcohol, it was extracted with this solvent in a Soxhlet extractor for 24 hours. The extract thus obtained was fractionated.

Fractionation

	Boiling Points	Remarks.
I	76- 78°	colorless ethereal odor.
II	78- 80°	" " "
III	80- 85°	colorless sulphur odor
IV	85-175°	very strong sulphur odor

The last fraction boiled constant at 105°c for some time but the final identification was impossible because the entire volume

of the fraction was but .5 cc and thus, as indicated by the boiling point, was anything but pure.

Further Study of the Mother Liquor.

The mother liquor from the black residue was saturated with dry hydrochloric acid as in the case of the gelatine. On cooling a very sparing crystallization took place. The process was repeated, placing the saturated alcoholic hydrochloric acid solution in a freezing mixture and keeping it there for 48 hours. The total weight of the crystalline deposit obtained was 19.5 gms.

This was found to be practically pure ammonium chloride instead of glycocoll hydrochloride, in fact the latter could not be isolated or definitely identified.

The mother liquor was then evaporated to a thick consistency and finally distilled under reduced pressure. Since no effective mercury pump was available, the ordinary Bunsen type water pump was used and hence no high vacuum could be obtained. An oil bath was used as the source of heat.

Fractionation

	Press.	Temp.	Wt.
I	28-30 mm.	35°-57°	182 gms.
II	40 "	57°-66°	30 "
III	70 "	66°-67°	23 "

All of the above fractions were fractionated under atmospheric pressure when two final fractions with following boiling points were obtained.

I	95°-102°	10.3 gms.
II	102°-106°	30.0 gms.

The first fraction proved to be simply a mixture of alcohol and water containing some hydrochloric acid.

The second fraction was refractionated, using a Galinski column when a constant boiling liquid distilled over at

106° = Boiling Point   20 gms.= weight

This last fraction contained a small amount of sulphur, some carbon and a great deal of hydrochloric acid. A volumetric determination showed 20% HCl (roughly) The hydrochloric acid was next neutralized with Na_2CO_3 and the mixture again distilled. It boiled at 99°C or the distillate consisted of water. From this the conclusion was drawn that this fraction originally consisted of a constant boiling mixture of hydrochloric acid in water.

Treatment of Residue from first Vacuum Distillation.

The residue from the first vacuum distillation was distilled under diminished pressure when a liquid boiling between 80 -175° C at 50 mm was obtained. This, however, was not studied farther.

At about 110°C, a yellow sublimate began to form in the neck of the distilling flask and increased in amount as the temperature rose. If the temperature was rapidly raised, a very small sublimate was obtained while non-condensible vapors were given off, indicating the decomposition of the sublimate. From about two-thirds of the total residue, 1 6.7 gms of crude sublimate was obtained. Since the crude substance was soluble in alcohol and insoluble in water, it was recryatallized from a mixture of equal parts of these solvents. The mother liquor was decolorized by boiling with charcoal and evaporated down to crystallization when the substances separated in small plates. A portion of this crystalline

deposit was saponified by boiling for about five hours with large excess of water in the presence of a little hydrochloric acid. On cooling crystalls separated out which were both insoluble in cold alcohol and water and were practically free from chlorine.

Melting point = 260°C.

An elementary analysis of this substance was made but owing to the small amount of the compound available,the carbon and hydrogen did not duplicate well. The results varied from 62-67 % in the case of carbon. Of all the amido-acids found in proteids,none except phenyl-alanine comes within these limits; the latter having a theroetical carbon content of 65,45%. Kjeldahl nitrogen determinations showed 8.75% the theroetical nitrogen content of phenyl-alanine being 8.45%.

The physical and chemical properties were then studied and found to correspond exactly to those of phenyl alanine.

Properties.

1. The substance melts at 260°C with decomposition.
2. The hydrochloride sublimes in needles.
3. The free substance (not the hydrochloride) is difficultly soluble in alcchol and water but readily soluble in ammonia.
4. It has a sweet taste.
5. It yields a copper salt difficultly soluble in water.
6. On heating rapidly it decomposes into an amine and carbon dioxide. From the analysis and the physical and chemical properties of this substance,it may be safely concluded that the compound under investigation is phenyl-alanine.

The presence of leucine was suspected but could not be absolutely proven although a substance having a very high melting point and most of the physical properties of leucine was isolated in small quantities.

On account of lack of time, other compounds present among the decomposition products of the beef proteids could not be isolated, although several distillates and residues which have not been worked with at all are at hand ready for further study when time will be available. Furthermore no attempt was made to determine quantitatively, the substances whose presence was definitely proven. The substances isolated are:

Melanoidic acid

Ammonia

Phenyl alanine

The presence of sulphur compounds of the sulphide or mercaptan type were manifested by the odor of the distillates and residues.

From the work done on the proteids of beef, the only conclusion that can be safely drawn is, that melanoidic acid, ammonia and phenyl-alanine are decomposition products of beef proteids as well as of the proteids hitherto studied. It may also be stated that sulphur in the proteid studied, is converted into sulphides or mercaptans on hydrolysis. The amount of time and attention given to these studies was of course insufficient and it would doubtless require a great deal of time before an accurate quantitative insight into beef proteids could be established.

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